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Review

Nutrients as trophic factors in neurons and the central nervous system: Role of retinoic acid

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In multicellular organisms, death, survival, proliferation, and differentiation of a given cell depend on signals produced by neighboring and/or distant cells, resulting in the coordinated development and function of the various tissues. In the nervous system, control of cell survival and differentiation is achieved through the action of a distinct group of polypeptides collectively known as neurotrophic factors. Recent findings support the view that trophic factors also are involved in the response of the nervous system to acute injury. By contrast, nutrients are not traditionally viewed as potential trophic factors; however, there is increasing evidence that at least some influence neuronal differentiation. During development the brain is responsive to variations in nutrient supply, and this increased sensitivity or vulnerability of the brain to nutrient supply may reappear during neuronal repair, a period during which a rapid membrane resynthesis and reestablishment of synthetic pathways occur. To further evaluate the potential of specific nutrients to act as pharmacologic agents in the repair of injured neurons, the effects of retinoic acid, an active metabolite of vitamin A, and its role as a trophic factor are discussed. This literature review is intended to provide background information regarding the effect of retinoic acid on the cholinergic phenotype and the differentiation of these neurons and to explain how it may promote neuronal repair and survival following injury. (J. Nutr. Biochem. 11:2–13, 2000)

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Introduction

Vitamin A is a micronutrient with an unusually wide biological scope of action including morphogenesis, vision, immune function, reproduction, neuronal development, and maintenance of differentiative functions.[1–3] For many years, its specific mechanisms of action, beyond those identified for vision, remained elusive. Recent identification of multiple nuclear receptors for the vitamin A metabolite retinoic acid (RA) has allowed a greater understanding of its myriad of cellular effects and its ability to modulate a broad spectrum of events.

During brain development, RA signals key events leading to the cessation of cell proliferation and terminal differentiation[4–7] and consequently can be considered a neurotrophin. Thus, although most neurotrophins are small, endogenously synthesized polypeptides, certain nutrients also play a trophic role developmentally. Notable among these nutrients are choline and RA. Reviews of the potential trophic role of choline are presented elsewhere and will not be addressed here.[8,9] Instead, attention will be given to the evidence that suggests a trophic role of RA and will focus

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on evidence that RA provides one of the signals directing the differentiation of those neurons destined to be cholinergic. Clearly, this is of fundamental importance with regard to neurodevelopment. More recent evidence, however, suggests that many compounds in neuronal differentiation may also play crucial roles following neuronal injury; that is, trophic factors appear to enable the repair of the injured neurons that they support during development.[10] This suggests that the same chemical environment may be required by the neuron during repair as during development, and opens the exciting possibility of aggressively treating brain injury with a view toward less functional loss.

To address this issue, general metabolism and mechanisms of action of RA will be addressed first. This will be followed by a separate examination of the antiproliferation and differentiation roles of RA in the developing central nervous system (CNS). Finally, evidence regarding the potential protective effect of RA following brain injury will be examined.

Postulated mechanism of action of RA

This section will briefly describe the mechanism of action and the various enzymes and proteins involved in RA biosynthesis and metabolism. Readers seeking more comprehensive information should consult several excellent reviews in this area.[11–13] The retinoids constitute a large family of natural and synthetic compounds that possess vitamin A activity or structural homology to retinol.[14] Vitamin A is a generic term designating any compound with β-carotene structure having qualitatively the biological activity of retinol.[15] Retinol is the transport form of vitamin A; retinal, or retinaldehyde, is the light-sensitive pigment for rhodopsin in the retina; and RA is the bioactive form involved in growth and differentiation.[3] β-Carotene in plants and retinyl esters in animal tissues are the two major dietary sources of vitamin A.[14] The first step in the conversion of β-carotene to vitamin A in the intestinal mucosa is an oxidative cleavage that produces all-*trans*-retinal, which is then reduced to retinol.[13] Retinol in the mucosal cell is re-esterified with long-chain mainly saturated fatty acids.[14] The retinyl esters, in association with chylomicrons, are then transported by means of the lymphatic system into the general circulation and are taken up by the liver by receptor-mediated endocytosis. Vitamin A is mobilized from liver stores and transported in plasma as retinol bound to retinol-binding protein (RBP).[13] RBP interacts with another plasma protein, transthyretin, and normally circulates as a 1:1 molar RBP-transthyretin complex.[14] Retinol, bound to RBP in the plasma, is effectively taken up by tissues, particularly by RA-dependent target tissues. The presence of a specific RBP receptor has been postulated for the uptake of retinol by cells.[11] However, the results on the characterization of specific RBP receptors are controversial. Two studies failed to detect specific binding of retinol to a RBP receptor.[16,17] The first study used primary mouse keratinocytes as a model system to compare the uptake and metabolism of [^{3}H]retinol delivered to them either free in solution or bound to RBP. No specific binding of ^{125}I-labeled RBP to monolayers of keratinocytes or membranes prepared from them was found, indicating the absence of a high-affinity RBP receptor on keratinocytes. The second study examined the uptake characteristics of retinol by liver parenchymal cells to assess whether retinol uptake is mediated by a cell-surface receptor for RBP. The [^{3}H]retinol uptake from [^{3}H]retinol-RBP showed a time-dependent increase and was not saturable at concentrations exceeding the physiologic concentration by more than a factor of 2. Uptake of [^{3}H]retinol was not inhibited by a 10-fold molar excess of unlabeled retinol-RBP. These data did not support the existence of a cell-surface receptor for RBP on rat liver parenchymal cells.

After delivery to the target tissue, retinol is either esterified into retinyl esters for storage or further metabolized into the biologically active RA.[11] Alternatively, RA can be taken from the RA pool present in the plasma. In plasma, RA circulates bound to albumin and within the cell it is bound to cellular RA binding proteins (CRABPI and II).

Several reports have suggested a model of retinoid metabolism that entails prominent roles for cellular retinoid-binding proteins (CRBP; see Ong[13] for a comprehensive review). CRBP binds retinol with high-affinity and participates in the enzymatic esterification of retinol into retinyl esters.[11,13] Both binding proteins (CRBP and CRABP) appear to play a critical part in facilitating the interaction of retinoids with binding sites on cell nuclei[14] and consequently play an active role in the intracellular distribution of the retinoids. Nevertheless, a more passive function might be to simply serve as a cellular pool of retinoid, thus keeping the concentration of free retinoid very low.[13] This would protect the relatively permutable retinoid from nonspecific reactions that might degrade it.[13] However, the physiologic importance of the CRABP was recently challenged by reports that mice deficient in CRABPI[18] and CRABPII[19] are normal and essentially indistinguishable from wild-type mice as judged by their normal development, fertility, life span, and general behavior. Moreover, CRABPI and CRABPII double mutant mice also appear to be essentially normal, and both CRABPII single mutant and CRABPI and CRABPII double mutant embryos are not more sensitive than wild-type embryos to RA excess treatment in utero.[19] Thus, it is clear that there is still much to learn about the various roles the CRBP play in the metabolism and function of retinoids.

The oxidation of retinol into retinaldehyde is catalyzed by a relatively nonspecific alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) can convert retinaldehyde into RA.[20] However, many studies have shown that ADH is not the only enzyme required for retinal and RA formation.[21,22] Napoli et al.[22] showed that a strain of deer mice lacking the ADH enzyme was still able to form retinal from retinol. This insight was complemented by the subsequent identification of microsomal retinol dehydrogenase (RoDH) isozymes, which were distinct from the cytosolic ADH isozymes. Once formed, the CRBP-retinol complex can also be esterified by a microsomal enzyme called lecithin:retinol dehydrogenase (LRAT) and the retinol converted into retinal by RoDH.[11] Additional cytosolic retinal dehydrogenase (RalDH) enzymes convert retinal into RA.[11] Therefore, the fate of RA between its synthesis and its metabolism and the exact enzymes involved in each of these processes still remain somewhat obscure. Most but not all

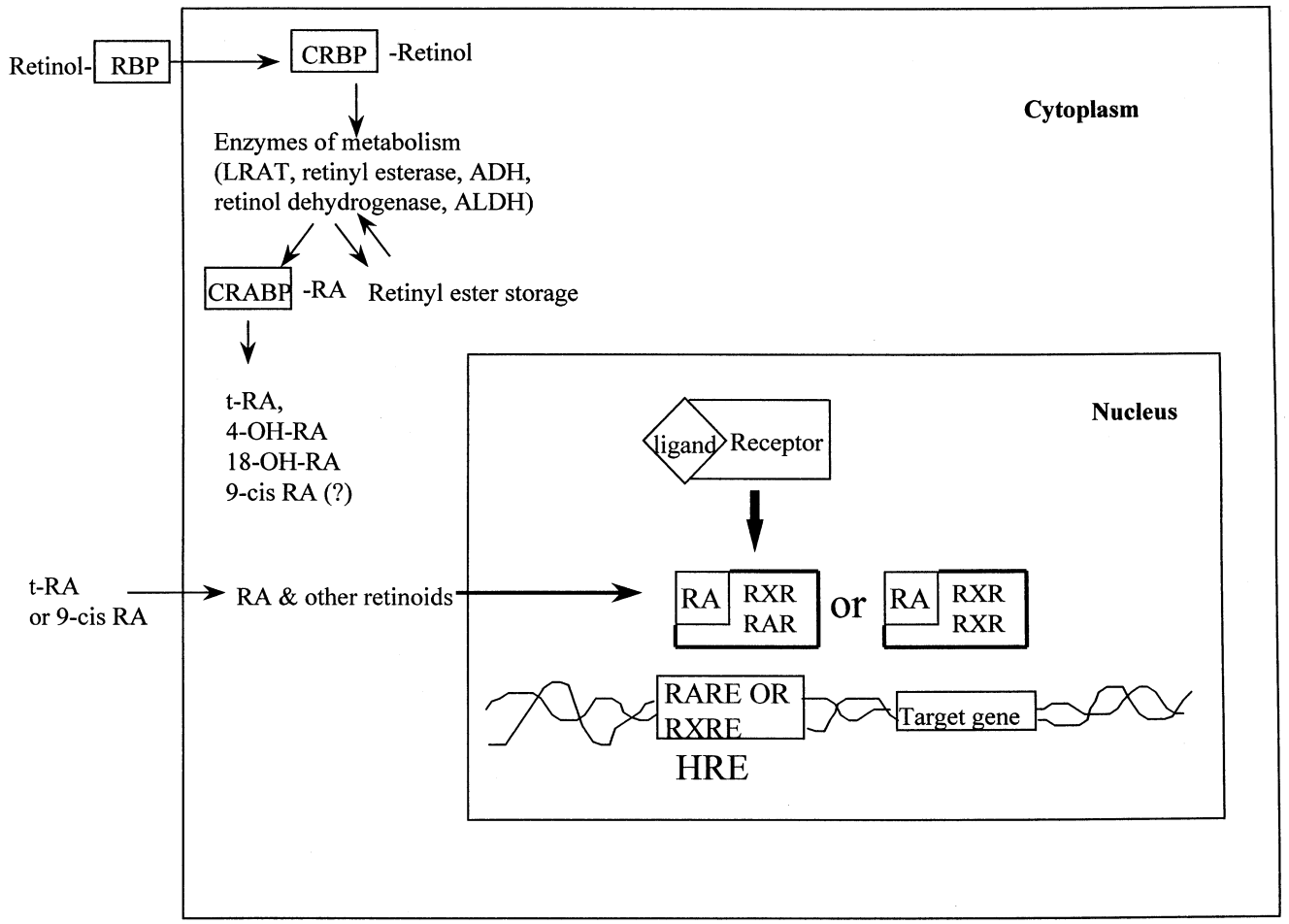

Figure 1 Overview of retinoic acid (RA) metabolism and mechanism of action. RA can act in both a paracrine and autocrine fashion by either entering the cell directly from plasma or being made from retinol and retinal in the target cell. Retinyl ester is the storage form of vitamin A. Metabolic activation of all-*trans* RA (t-RA) is a multistep process, involving many enzymes. Retinol bound to retinol-binding protein (RBP) in the plasma is effectively taken up by the cells and converted to RA, which then binds to its receptors [retinoid X receptors (RXR) and/or RA receptors (RAR)] within the nucleus. The ligand receptor complex modulates gene expression by binding as homo- or heterodimers to specific DNA sequences known as RA response elements (RARE or RXRE) located in the promoter region of the target gene. CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid binding protein; LRAT, lecithin:retinol aceyltransferase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; HRE, hormone response elements. (Adapted from Wuarin et al.[7])

cells seem to have the machinery needed to produce RA and contain low concentrations of this retinoid.[23,24] Thus far, at least five naturally occurring, biologically active retinoids have been identified. These are all-*trans* RA (t-RA),[25–27] 3,4-didehydroretinoic acid,[28] 9-*cis* RA (9-*c*RA),[29,30] all-*trans* 4-oxo RA,[31] and 14-hydroxy-4,14-retroretinol.[32] It is not clear at the present if isomerization of t-RA to 9-*c*RA is a crucial step in the bioavailability of 9-*c*RA (*Figure 1*). One in vivo study failed to observe bioisomerization of t-RA to 9-*c*RA, indicating that some other mechanism may be involved in its synthesis.[33] In addition to being synthesized intracellularly, 9-*c*RA also can be delivered to the cell directly from the plasma.[33] However, in vivo plasma levels of 9-*c*RA are much lower than levels of t-RA. In humans, 9-*c*RA concentrations are below the detection limit of 1 nM.[34] Endogenous levels of 9-*c*RA in whole rat embryos also were below limits of detection but small quantities of this isomer could be detected in the neonatal rat eye and human embryonic brain.[35] One study reported the identification of a stereospecific 9-*c* retinol dehydrogenase, which is abundantly expressed in embryonic tissues known to be targets in the retinoid signaling pathway.[36] The membrane-bound enzyme is a member of the short-chain ADH/reductase superfamily, and is able to oxidize 9-*c* retinol into 9-*c* retinaldehyde, an intermediate in 9-*c*RA biosynthesis. Analysis by nonradioactive in situ hybridization in mouse embryos revealed prominent expression of the enzyme in parts of the developing CNS. The identification of this enzyme reveals a pathway in RA biosynthesis, where 9-*c* retinol is generated for subsequent oxidation to 9-*c*RA. When treated with 9-*c*RA, P19 mouse embryonal carcinoma cells differentiated into neurons and glial cells.[37] These findings support an intriguing possibility that the 9-*c*RA/retinoid X receptor system may play an important role in neural differentiation. Interconversion of the t-RA and 9-*c*RA can provide

a novel means for cell-specific regulation of the activity of the two retinoid pathways.

The mechanism by which retinoids are able to elicit their diverse effects ultimately resides in their ability to regulate gene expression via a receptor-mediated event.[38] These receptors are nuclear-acting transcription factors that become active after binding to their related ligands. The ligands for these receptors are lipophilic molecules, which include the steroid/thyroid hormones and the fat-soluble vitamin A metabolites and 1,25-dihydroxyvitamins D_3.[38] Three main subtypes of mammalian RA receptors (RAR) have been identified: RARα, RARβ and RARγ. In addition, a second class of retinoid-responsive transcription factors—the retinoid X receptors (RXR)—have also been discovered, which also possess three subtypes: RXRα, RXRβ and RXRγ.[39,40] Upon ligand binding, the RARs and RXRs bind as dimers to hormone response elements (HRE) in the promoter region of target genes to enhance or repress transcription (*Figure 1*).[41] Although the RARs and RXRs are the only receptors that directly interact with or bind retinoids, an increasing number of other receptors are being identified that interact with the retinoid receptor either by heterodimer formation or by competing for the same site on the DNA. These proteins have been referred to as RAR-related orphan receptors (ROR).[42] The specific function of the RORs has not yet been identified.

HREs are specific DNA sequences in the promoter region of the target genes, and two such response elements have been identified for RA receptors: RAR and RXR response elements (RARE and RXRE, respectively).[41] Diversity in RA effects appears to be generated by heterodimeric interactions between these two families of nuclear RA receptors and by the existence of these polymorphic RA response elements.[38] The identification of two (and potentially three) retinoid receptor systems (RAR, RXRs, and probably ROR) and two distinct retinoid hormones (t-RA and 9-*c*RA) has allowed an updated version of the retinoid signaling pathway to be considered (for excellent reviews in this area see Mangelsdorf[38] and Pfahl and Chytil[12]).

RA and related vitamin A derivatives exert profound effects on many biological processes, including embryonic pattern formation, cell proliferation, and differentiation.[1] As stated earlier, one explanation for the diversity of the retinoids' actions is the multiplicity of its nuclear receptors. Most tissues in the body are sensitive to the nuclear effects of RA. Nevertheless, of particular interest here is the effect of RA on the CNS and its ability to regulate key events including proliferation and differentiation during development and in the mature neuron. Evidence has shown that the embryonic CNS requires vitamin A and its derivatives, particularly RA, for its proper development.[2] Both excess[43–45] and a deficiency[46] of RA cause abnormal development of the CNS. Moreover, RA itself as well as cytoplasmic proteins that specifically bind RA and the nuclear receptors for RA are all expressed in precise domains and neuronal cell types within the CNS.[1] Thus, distinct regional and cellular distributions support the contention that RA is involved in the development and maintenance of the CNS. The two key events during development of the CNS are proliferation and differentiation. Therefore, to address the role of RA within the CNS, it is important first to understand the interrelationship between signals associated with proliferation and differentiation.

Role of proliferation versus differentiation signals in development of the CNS

There is mounting evidence of an important relationship between regulation of cell proliferation, CNS pattern formation, and neural differentiation.[47] The nervous system is derived from multipotential precursor cells that show a closely regulated inverse relationship between cell proliferation and differentiation[48]: The bulk of morphologic differentiation of neuronal progenitors occurs after cells have ceased proliferating.[48] Nevertheless, several observations suggest that cell-cycle regulation directly impacts on differentiation. Thus, in the developing CNS, multipotent stem cells do not respond to environmental differentiation factors until their final rounds of division.[48] However, the molecular interaction that coordinates cell-cycle regulation with CNS pattern formation and neural differentiation remains enigmatic.

Differentiation is defined as "a set of heritable factors that 'commit' the cell to a certain type, as well as environmental influences that allow the expression of a unique phenotype."[49] Morphologic and biochemical differentiation of neuronal cells can be induced by several conditions and compounds. This is most often examined in tissue culture by growing cells in the absence of serum or the presence of growth and trophic factors such as RA.[49]

Differentiation results in the loss of the ability of neuronal cells to proliferate. Although this property of adult neurons is probably critical to maintaining the integrity of the CNS, it results in the absence of regeneration of damaged areas of the brain. In contrast, tissue injury can trigger a proliferative response in other tissues such as liver. This leads to the conclusion that mammalian CNS neurons express a phenotype that is programmed to irreversibly shut down the cellular responses to mitogenic signals. However, recent in vitro and in vivo studies have indicated that neural stem cells with self-renewal and multilineage potential are present in the adult mammalian forebrain and neurogenesis continues throughout life.[4,50–52] Both RA and neurotrophins play important roles in neurogenesis during development but their roles in the adult CNS are not completely understood.

Due to limited mitogenic potential in adult neurons, reestablishing existing developmental differentiation pathways may become an important step in the repair of injured neurons. It has been shown that following injury neurons undergo a biphasic response in which expression of phenotypic macromolecules (neurotransmitter production examined almost exclusively) is initially reduced and production of those proteins involved in repairing the cytoskeleton is enhanced. Following morphologic repair, reestablishment of neurotransmitter production occurs.[53–55] During this period, specific neurotrophins reappear and indeed survival of injured neurons can be modulated by optimal trophic factor conditions.[56,57] Hence, those signals that drive the

original differentiation of the cell appear to be essential in assisting injured neurons in reestablishing their phenotype. Not surprisingly, a concerted effort is being placed on the identification of those trophic factors that provide differentiation signals developmentally and also can serve to support repair processes. Due to its effects on both neuronal proliferation and differentiation, RA is a candidate for examination.

RA's effects on proliferation

RA is recognized as having a strong anti-mitogenic effect in a variety of cell types.[3,58] Although the effects of retinoids on the proliferation of normal cells appear variable, these molecules inhibit the growth of cells treated with a tumoral promoter.[58] It has been proposed that RA may inhibit proliferation either by promoting the differentiation of proliferating cells or by directly inhibiting cell division by blocking the G1/S phase of the cell cycle.[58] The majority of work in this area has been conducted in malignant cells as a means of determining the usefulness of RA in the treatment of certain cancers. To date only one study has attempted to determine the effects of RA in a neuronal cell system. Salvarezza and Rovasio[59] observed that RA decreases the proliferative activity of neural crest cells both in vitro and in vivo.

In contrast, the vast majority of work has been conducted in neuroblastoma cell lines. Neuroblastoma is one of the most common pediatric solid tumors, frequently occurring in infancy, with the primary lesion in the adrenal and sympathetic chains.[48] These tumors can spontaneously differentiate into benign ganglioneuroma containing neurons and Schwann cells.[60] Some neuroblastoma cell lines can be induced to differentiate in vitro into cells resembling neurons and many lines have been used as models to determine what types of factors induce differentiation and therefore may be clinically beneficial. For example, many human and murine neuroblastoma cell lines have been developed that display properties associated with differentiated adrenergic or cholinergic neurons, including expression of key phenotypic enzymes involved in neurotransmitter synthesis.[61–64]

N-myc expression is associated with metastatic disease, as well as the undifferentiated state of normal neuroblasts migrating from the neural crest during embryogenesis.[65] It has been hypothesized that constitutive expression of N-myc inhibits exit from the cell cycle and blocks neuronal cell differentiation.[66] Recent findings suggest that N-myc is involved in regulation of neuronal differentiation in the neural crest cell population.[67] However, whether N-myc promotes growth and/or antagonizes neuronal differentiation of neuronal cells or whether the down-regulation of N-myc occurs as a consequence of the onset of differentiation have not been determined. The ability of RA to induce differentiation in several types of human and murine tumor cells also is associated with the ability of RA to suppress the expression of either the c-myc oncogene or the related N-myc gene.[68] Cell differentiation induced by RA results in morphologic changes characteristic of the mature neuronal phenotype, including outgrowth of neurite-like structures with several interconnections.[69] Down-regulation of N-myc gene expression by RA has been shown in a number of cultured neuronal and neuroblastoma cells.[70–72] Therefore, differentiating effects of RA may lead indirectly to an increase in neuronal survival. However, there is currently no evidence supporting this hypothesis.

A number of researchers have suggested a correlation between increasing cyclic adenosine monophosphate (cAMP) levels and negative cell growth and differentiation in a variety of tumor cell lines.[49] However, it is not the actual levels of cAMP that have an effect on cell proliferation. Rather, it is through the cAMP-activated protein kinases and subsequent modification of these proteins that cell responses are regulated.[49] RA has been shown to increase cytosolic and plasma membrane-bound cAMP-dependent kinase activity in embryonal carcinoma cells.[49]

RAR has been linked to the regulation of human neuroblastoma cell growth. Plum and Clagett-Dame[73] showed that both 9-*c*RA and t-RA were equipotent in inhibiting the growth of human neuroblastoma cells and inducing RARβ mRNA. The argument presented was that, because both isomers were equally effective in inhibiting cell proliferation and inducing RARβ, this was the most likely underlying mechanism by which these compounds were eliciting their specific effect on cell growth.

Proliferating cells such as neuroblasts may require growth factors not only to initiate mitosis, but also to coordinate successful passage through the cell cycle; that is, if traversal of the cell cycle is initiated, but not coordinated, by appropriate growth factors, apoptotic death occurs. Furthermore, cells that are out of the cell cycle (i.e., in G0), such as postmitotic neurons, are protected from apoptotic death by the presence of trophic support.[74] Therefore, during development, the role of agents such as nerve growth factor (NGF) and RA may be to protect neuronal cells from apoptosis either by causing them to withdraw from the cell cycle or by preventing them from reentering the cycle. In other words, RA may promote survival of developing neurons first by preventing their proliferation and second by triggering their differentiation.

The focus of neuronal repair is to restimulate expression of certain key enzymes and trophic factors that are down-regulated following neuronal injury and to reinitiate signaling pathways.[56] How is RA able to play a trophic role in these postmitotic cells and promote their survival? One simple hypothesis might be that RA triggers a signal transduction pathway in neuronal cells that leads toward cellular differentiation and survival of terminally differentiated G0 arrested cells.

As already mentioned, one fundamental step in terminal differentiation is arrest of cellular proliferation. This has led many researchers to suggest that cellular proliferation and differentiation involve two alternate antagonistic cellular programs: Induction of a proliferative pathway will lead to inhibition of a differentiation pathway and vice versa. In many disorders of the CNS, disability accumulates as a result of the degenerative process and the failure to repair. Hence, agents such as RA that promote differentiation may be candidate compounds in supporting nerve repair following injury. Like many of the trophins, RA shows a certain degree of specificity in terms of promoting differentiation of various neuronal phenotypes.[75–78] Although the focus of this review is the effect of RA on the cholinergic phenotype,

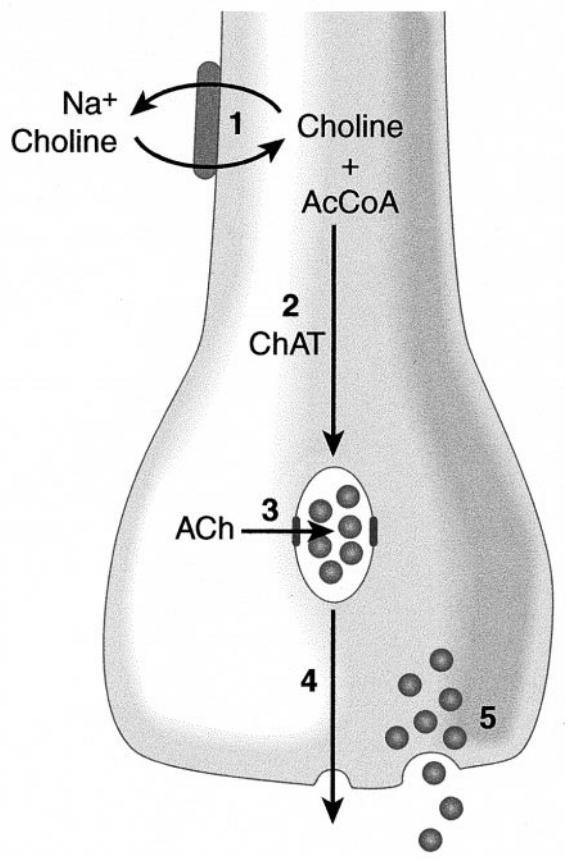

Figure 2 The cholinergic nerve terminal: 1, sodium dependent high-affinity choline uptake; 2, choline acetyltransferase (ChAT); 3, acetylcholine (ACh) transporter; 4, exocytosis of the vesicle; 5, ACh leakage.

other neuronal subpopulations are also sensitive to RA; for example, dopaminergic neurons show reduced expression of the dopamine receptors in the absence of retinoid receptors,[79] RA is known to induce neural tissue-type differentiation in various preparations including embryonic carcinoma cell lines[80–82] and embryonic stem cells.[83] RA was also shown to induce neuron formation by immortalized neuroectodermal progenitor cells derived from the brain vesicles of 9-day-old mouse embryos that lacked a functional p53 tumor suppressor gene.[84] However, the particular interest here is the effect of RA on cholinergic neurons and its ability to regulate expression of associated phenotypic proteins.

Cholinergic system

Neurons that synthesize, store, and secrete the neurotransmitter acetylcholine (ACh) are designated cholinergic.[85] The cholinergic phenotype encompasses proteins that allow the cell to synthesize, store, release, uptake, and degrade its neurotransmitter ACh.[85] The cholinergic system in the CNS consists of several areas that are localized throughout the brain and spinal cord. These neurons are identified by the presence of choline acetyltransferase (ChAT), which is the enzyme involved in ACh synthesis, and the accumulation of choline, which is one of the substrates for ACh synthesis, by a high-affinity, sodium-coupled choline transporter (*Figure 2*). Once synthesized, ACh is stored in secretory vesicles with the assistance of vesicular ACh transporter (VAChT). Following release, the action of ACh is terminated by cholinesterases. The most effective of these is acetylcholinesterase (AChE), which breaks ACh down into choline and acetate. Approximately half of the choline thus released is taken back up by the presynaptic neuron to be reformed into ACh.[85] Although many neurotrophins have been implicated as differentiation signals for cholinergic neurons,[86–90] the next section will deal with some of the effects of RA on phenotypically distinct cholinergic neurons.

Role of RA in the differentiation of cholinergic neurons

Establishment of a transmitter phenotype is an essential step in neuronal development. The choice of transmitter phenotype includes induction of a combination of specific genes encoding transmitter-related enzymes (synthesizing and degrading enzymes) and a number of precursors and transport molecules. The decision of the transmitter phenotype of a particular neuron during development is known to be made not only on the basis of an endogenous cell program but also to be influenced by environmental factors.[76,91–95] Although little is known about the role of retinoids in the development of cholinergic neurons, the effects of RA on the cholinergic properties of several neuronal culture systems have been examined in vitro. These results suggest that maintenance of septohippocampal projections in vivo may be influenced by retinoids.[96]

It is well known that the regulation of ChAT activity under physiologic and pathologic conditions is important for the development and neuronal activities of cholinergic systems involved in many fundamental brain functions. A deficiency in ChAT activity has been reported in many neurodegenerative diseases (e.g., Alzheimer's disease and motor neuron disorders).[97–101] This section will focus on how RA can effect ChAT synthesis and expression either directly or alternatively by indirect effects on cholinergic differentiation by promoting the expression of other neurotrophic factors that effect the cholinergic phenotype.

Direct effects of RA

Cholinergic neurotransmission depends on the coexpression of proteins involved in the synthesis, storage, and release of ACh.[75] A number of studies have shown that RA treatment results in biochemical differentiation of cholinergic neurons in a variety of established cell lines and primary preparations in vitro.[64,76,93,102,103] In addition, it has been shown that these differentiation-promoting effects of RA are cell- and phenotype-specific. For example, the specificity of action of RA on ChAT in PC12 cells was strengthened by the observation that the activity of another transmitter-related enzyme, glutamic acid decarboxylase (GAD), was not effected by RA.[103] A similar study showed that RA increased the survival and biochemical differentiation of cholinergic neurons in vitro without modifying those of GABAergic neurons.[104] These results suggest that RA exerts specific differential trophic effects on various neuro-

nal populations. The differential and promoting effects of RA on cholinergic neurons were supported by biochemical determinations of ChAT and AChE activities, which showed that RA increased total and specific activities, whereas GAD activity, the biosynthetic enzyme for GABA, was unaffected by RA treatment. RA treatment also increased the overall survival of cholinergic neurons. Immunocytochemical quantification of cholinergic and GABAergic neurons demonstrated increases in the number of ChAT positive cells, whereas the number of GABA positive neurons was not altered after RA treatment.[104] Two different cell types, including neuroblastoma cells and cultured rat sympathetic neurons, can be induced to differentiate into cholinergic neurons by RA.[76,96] For example, when induced with RA, LAN-5, a human neuroblastoma cell line, differentiates exclusively into cholinergic neurons expressing cholinergic markers.[105]

Of the proteins contributing to the cholinergic phenotype, the best studied to date has been the ACh synthesizing enzyme ChAT. ChAT activity and expression have been used as markers for cholinergic neurons and as indices for the action of trophic factors on these neurons. Previous studies have shown that ChAT activity and expression can be modulated by RA.[64,76,93,102,103] Treatment with RA increases ChAT activity in cultures of rat embryonic brain,[106] sympathetic neurons,[76,93] rat pheochromocytoma PC12 cells,[103] human neuroblastoma NB69,[107] MC-1XC,[102] and LA-N-2 cells.[64] However, the mechanism leading to the RA-induced increases in ChAT activity remains uncertain. To this end, the effects of retinoids have been extensively studied in the SN56 cholinergic neuroblastoma cell line.[96] It was reported that retinoids increased ChAT mRNA levels, ChAT activity, and intracellular ACh concentration.[96] Moreover, it was shown that ACh levels could be enhanced by concentrations as low as 1 nM,[96] suggesting that the effect is physiologically relevant for septal neurons in vivo. The fact that ACh levels in SN56 increased as early as 6 hours after treatment with RA, together with the presence of putative RAREs in the murine ChAT gene, indicate that RA may act directly in ChAT gene expression.[96] It is not yet known whether any of the RAREs present in the 5′ region of the ChAT gene constitute true response elements.[96] Both t-RA and 9-*c*RA were equally effective at enhancing the cholinergic phenotype of SN56 cells, suggesting that the isomerization of t-RA to 9-*c*RA did not occur and that the observed effects were direct effects of each isomer. Furthermore, the retinoids appeared to modulate these effects by activation of RARα.[96] This suggests that the regulation of cholinergic gene expression by RA is mediated by a different set of receptors (namely, RARα) than its effect on cell growth (namely, RARβ). These RA receptor genes are expressed with varying degrees of tissue specificity during embryonic development and in adult tissues and may regulate different events in different tissues.[1,3,39,108] This may account for how RA could have a general effect on proliferation, but a more specific effect on cholinergic differentiation.

The most likely explanation for the increased abundance of the ChAT mRNA levels in the t-RA-treated SN56 cells is accelerated rate of transcription of the ChAT gene. This hypothesis is consistent with the well-described actions of the t-RA receptors as *trans*-acting transcriptional regulators. Indeed the 5′ region of the mouse ChAT gene contains a nucleotide sequence (GGTTCACATGTTCA) that closely resembles the consensus sequence of the RA-responsive elements (which often include a GGTTCA repeat).[109]

The effects of RA are not limited to ChAT and could be extended to other proteins that are considered components of the cholinergic phenotype. One such protein, VAChT, has recently been shown to be induced by RA.[110] ChAT and VAChT proteins are encoded by two closely linked genes in vertebrates, with the VAChT coding sequence contained within the first intron of the ChAT gene.[110] This unusual genomic organization suggests that the transcription of these two genes is coordinately regulated. Consistent with this prediction, it was reported that the mRNA levels for both VAChT and ChAT were increased by retinoids.[110] It would make sense that the expression of these two proteins would be linked so that the ACh that is synthesized in the cell could be protected from intraneuronal degradation.

Although the majority of studies point toward an increased transcription of ChAT being the underlying mechanism by which RA increases intracellular ACh, there are some dissenting views. For example, one study showed that α-amanitin, which is an inhibitor of transcription, did not abolish the stimulatory effects of t-RA on ChAT activity in a human neuroblastoma cell line.[102] In addition, inhibitors of translation also failed to attenuate the elevations in ChAT activity caused by t-RA. Therefore, it was concluded that t-RA caused ChAT activation by a post-translational process; however, the exact mechanism of action was not determined.[102] Although post-translational modification of the ChAT gene may partly account for the mechanism of effect of t-RA, it is clear that the bulk of evidence points toward a transcriptional effect of RA.

Indirect effects of RA

In addition to the direct effect of RA on cholinergic phenotypic expression, it may also act by enhancing the neuron's response to other trophic factors.[4] Neurotrophins applied to RA pretreated cells do not effect the number of neurons generated but accelerate a neuron's acquisition of mature transmitter phenotype.[4] Most commonly observed is an effect of RA on neurotrophin receptor availability. For example, RA has been shown to up-regulate ciliary neurotrophic factor (CNTF) receptors in cultured chick embryos and cardiomyocytes.[111] Although RA binds to nuclear retinoid receptors and CNTF/leukemia inhibitory factor (LIF) binds to specific receptors associated with the plasma membrane, these two compounds may share a common downstream mechanism of inducing cholinergic properties. Therefore, levels of retinoids in tissues and/or RA receptors in cells may serve as a switch for cells to enhance their sensitivity to CNTF by increasing synthesis of new receptor protein. The increased CNTF response in developing neurons could result in, for example, enhanced cholinergic differentiation or cell survival.

Trophic factors belonging to the CNTF/LIF family and cAMP also enhanced VAChT and ChAT mRNA levels in SN56 neuroblastoma cells.[110] Significantly, this up-regulation resulted in proportional increases in the intracellular

ACh levels. The effects of these agents were additive with respect to RA, pointing to several independent mechanisms by which the cholinergic properties of septal neurons can be modulated.[110] Clearly, these data do not support a combined effect of RA mediated through a combination of a direct role of RA and increased CNTF responsivity secondary to elevated receptor availability. Whether this lack of synergism between CNTF and RA is specific to the SN56 cells or more applicable to other cholinergic neurons is unknown.

More compelling data for an indirect effect of RA on cholinergic expression comes from studies of NGF. NGF appears to play an important role in maintenance of sympathetic and sensory neurons in the peripheral nervous system and cholinergic neurons in the CNS.[112] Chronic intracerebral administration of NGF to adult or aged rats leads to enhanced expression of cholinergic neuronal phenotypic markers and increased ACh synthesis and release.[113–116] RA has been reported to increase the expression of NGF receptors.[117–119] Therefore, RA may induce cholinergic differentiation indirectly by promoting NGF receptor expression.

Other researchers have suggested that the effects of RA on the activity of ChAT in certain murine cells may be indirect and mediated by midkine, the 13-KDa product of a RA responsive gene and a member of a family of heparin-binding growth factors.[120] Midkine has been reported to mediate the induction of ChAT activity by RA in a murine p19 embryonic carcinoma cell line and to stimulate ChAT activity in cultured mouse fetal spinal cord neurons.[121,122] Treatment of p19 cells with anti-midkine antibody resulted in the reduction of ChAT activity to 40% of that observed in cells treated with RA alone. However, ChAT activity in anti-midkine plus RA-treated cells was still significantly higher than that observed in the control cells, which suggests that midkine may only account for part of the induction in activity.[122]

In summary, numerous studies provide evidence that RA exerts a direct effect on the expression of a variety of cholinergic-specific proteins. This effect is most likely mediated via the nuclear RAREs. Furthermore, RA may enhance the expression of receptors for other neurotrophins involved in the differentiation of cholinergic neurons. Nevertheless, co-culture of neuronal cells with CNTF or NGF and RA do not provide evidence for the anticipated synergistic response.

Nutritional impact of vitamin A

In addition to its role in development and morphogenesis, new physiologic functions of RA have been identified. These include its role in immune defense reducing morbidity of measles and respiratory and possibly human immunodeficiency virus (HIV) infections and in cell growth and differentiation, and its potential role as a neurotrophic factor.[1,123–126]

Recent studies have shown that retinoids play significant roles within the CNS, both during development and in the adult. One such study showed that vitamin A nutritional status differentially regulates the expression of RAR in rat tissues.[127] In addition, retinoid deficiency and abnormal retinoid metabolism have been implicated in neurodegeneration associated with aging and with certain brain pathologies such as Alzheimer's disease.[128,129] Among the RARs, RARβ is the main isoform expressed in the mature brain and this receptor is up-regulated by RA.[130] It has been shown that RARs are under-expressed in the older brain and in the Alzheimer's diseased brain.[130] However, it has been suggested that the level of RAR expression can be restored by retinoid administration.[128]

Research to date is promising but the question of how much vitamin A is needed for possible protective and beneficial effects remains unanswered. Most of the research defining the unique features of vitamin A does not contribute to determining human dietary requirements except to caution against too high or too low an intake. The recommended daily intake in the United States for healthy males is 1,000 mg retinol equivalents (RE)/day and 800 mg RE/day for healthy females.[15] The analysis of dietary needs is complicated by the fact that a variety of mechanisms tightly control uptake, storage, release, and metabolism of vitamin A. A better understanding of vitamin A metabolism and how it is regulated would provide insight into disease states in which abnormal vitamin A function is postulated.

Current knowledge, injury, repair, and disease

From the above-mentioned analysis emerges the general principal that the signals transduced by cells during growth and physiologic activity are the same as those that become overloaded during pathologic events and aging. In other words, a role for neurotrophins in mature neurons persists, extending beyond that of promoting survival during development to one of maintaining phenotypic and functional properties. In the context of the nervous system, the extent to which a neuron can survive is modulated by its trophic factor dependent state of health. Conversely, optimal neurotrophic factor levels may save neurons from otherwise lethal events.

Many adaptive changes that occur after neuronal injury are believed to be influenced by altered availability of neurotrophic factors to the neurons in that state. For example, it has been hypothesized that some neurodegenerative disorders, including Alzheimer's disease, could be due to a deficiency in endogenously produced trophic factors or to decreased responsiveness of neuronal populations to these agents. Indeed some studies have suggested an association between Alzheimer's disease and abnormal retinoid metabolism.[129,131] This in turn may lead to a deficiency in RA levels that are available intracellularly.

In addition to RA, the roles of other neurotrophins during neuronal repair has been investigated; among these are NGF, CNTF, LIF, brain-derived growth factor (BDNF), and fibroblast growth factor (FGF).[112] Although sometimes classified as neurotrophins, these pleiotrophic molecules also effect non-neuronal cells.[112] Of these, the most well-known neurotrophic factor known to affect neuronal survival is NGF. NGF appears to be important in maintaining phenotypic and functional properties associated with the intact axonal state and reestablishing these properties after pathologic insult. It has been shown that administration of exogenous NGF counteracts many degenerative changes observed in the subpopulation of axotomized neurons,

which are NGF responsive.[57,113,132] CNTF can also act as a survival factor for motor neurons. Both CNTF and LIF promote cholinergic differentiation of sympathetic neurons and influence survival of motor and sensory neurons. Axotomy of facial motor neurons in newborn rats normally results in death of the majority of the motor neurons, but local application of CNTF to the injury site has achieved almost complete rescue of the cholinergic motor neurons.[133,134] This was one of the first indications that a neurotrophic factor may act in vivo to support motor neuron regeneration. Similarly, BDNF and FGF protect a range of hippocampal, septal, and cortical neurons from hypoglycemic and hypoxic injuries, and their tissue expression increases following ischemic and other insults in vivo.[56]

To date, this line of investigation has not examined the impact of RA administration following neuronal injury. Nevertheless, the similarities between the mechanisms of action of the more traditional neurotrophins with those of RA suggest that this retinoid could play a similar role. A search for pharmacologic therapeutic agents that may promote neuronal survival in the early degenerative and regenerative stages following neuronal injury would be useful adjuncts to the use of trophic factors.

Summary

RA and a number of other trophic factors are involved in cholinergic differentiation. Yet to be determined is how the interactions between these various factors effect cholinergic properties and whether it is through a common underlying pathway. By better understanding the relationship between neurotrophic factors and neuronal injury, it may be possible to facilitate the regeneration of injured neurons in a way that is beneficial to functional recovery.

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